DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 p24 ANTIGEN
Coulter HIV-1 p24 Antigen Assay
(Murine Monoclonal)
Immune Complex Dissociation

I. PRINCIPLE

The Human Immunodeficiency Virus Type 1 (HIV-1) is recognized as the etiologic agent of acquired immunodeficiency syndrome (AIDS). The virus is transmitted by sexual contact, exposure to infected body fluids or tissues, and from mother to fetus or child during perinatal period. After exposure to the virus, HIV-1 infection is characterized by an early period of antigenemia in which HIV-1 antigens (Ag) are detectable in blood. In most individuals the antigen level becomes undetectable for a period of time; late in disease, increasing failure of the immune system and increasing levels of virus may again result in detectable levels of antigen. One of the viral components in blood during antigenemia is the core protein, p24, the major internal structural protein of HIV-1.

The ICD HIV-1 antigen assay is a modification of the standard HIV-1 antigen assay. Circulating HIV-1 antigen binds to native HIV-1 antibody to form an immune complex and that is hidden from detection by the standard assay. The dissociation of the immune complex, accomplished by pH and heat, allows the p24 antigen to become detectable by the routine assay.

The Coulter HIV-1 p24 Antigen Assay is an enzyme immunoassay (EIA, or Enzyme-linked Immunoabsorbant Assay, ELISA) developed for detection and quantitation of the HIV-1 p24 core protein. The Coulter HIV-1 p24 Antigen Assay uses a murine monoclonal antibody to HIV-1 p24 antigen coated onto microtiter strip wells. A specimen of plasma or serum and lysis buffer are added to a coated well and incubated. If present, the viral antigen binds to the monoclonal antibody to the microtiter well. Following a wash step, biotinylated human anti-HIV-1 IgG is added to the well and, during incubation, binds to any HIV-1 p24 antigen bound to the well. Following another wash step, streptavidin-horseradish peroxidase is added which complexes with biotinylated antibodies. In a final step, a substrate reagent containing tetramethylbenzidine (TMB) and hydrogen peroxide is added which reacts with complexed peroxidase to form a blue color. The reaction is terminated by the addition of acid, and the absorbance is measured spectrophotometrically. The intensity of the color is directly proportional to the amount of uncomplexed p24 antigen in the plasma or serum. The quantity of HIV-1 p24 antigen in a specimen is determined by comparing its absorbance with that of known HIV-1 p24 antigen standards.

II. SPECIMEN REQUIREMENTS

Serum or plasma collected in acid-citrate-dextrose, (ACD), citrate-phosphate-dextrose with adenine (CPDA-1), EDTA, sodium citrate or heparin may be used and should be tested as soon as possible following collection. If the situation limits the ability to test the sample quickly, the

specimen can be held in refrigeration (2-4^{0}C) for a maximum of 7 days. If the period of time will be greater, the sample can be held at -20^{0}C or -85^{0}C for long term storage.

Remove the serum from the clot, or plasma from the red cells as soon as possible to avoid hemolysis.

Heat-inactivated specimens or specimens with obvious microbial contamination are unacceptable.

Specimens containing particulate matter may give inconsistent results. Such specimens should be clarified by centrifugation prior to assay.

Avoid subjecting specimens to repeated freeze thaw cycles.

Bring all specimens to room temperature (15-30^{0}C) prior to assay.

III. REAGENTS

A. Reagents included in Coulter HIV-1 p24 Antigen Assay Kit, 96 (PN 6604534) or 2400 (PN 6607051), include the following:

1. HIV-1 p24 Antibody-coated Microtiter Srtips. Store at 2-8^{0}C. Note manufacturer's outdate.

    a. Bring pouch containing HIV-1 p24 antibody coated microtiter strips to room temperature (15-30^{0}C) before opening to avoid condensation on the strips.

    b. The plate consists of 12 removable strips of 8 wells each. Any partial use of strips commits all 8 wells to the assay. Antibody coated strips may be used only once. When using a 96 well plate washer and fewer than 12 strips are needed, place uncoated strips in the remaining positions.

    c. Unused strips may be placed back into the pouch and sealed with the desiccant provided and stored at 2-8^{0}C for 60 days.

2. Anti-HIV (human)-Biotin Reagent. Store at 2-8^{0}C. Note manufacturer's outdate. The reconstituted reagent is stable for 2 months. Bring to room temperature (15-30^{0}C) prior to assay.

    a. Add 21 mL of distilled water to the Biotin Reagent vial and recap the vial.

    b. Gently invert vial to mix contents. Allow 5 minutes for the contents to dissolve.

3. Normal Human Serum (NHS). Store at 2-8^{0}C. Note manufacturer's outdate.

4. SA-Buffer (Tris buffer for SA-HRPO). Store at 2-8^{0}C. Note manufacturer's outdate.

5. SA-HRPO (Streptavidin conjugated to horseradish peroxidase). Store at 2-8^{0}C. Note manufacturer's outdate. Prepare SA-HRPO Working Dilution as follows:

   a. Within 15 minutes prior to use prepare the SA-HRPO Working Dilution. For a complete 96 well plate, add 21 µL of SA-HRPO reagent to 21 mL of SA-HRPO Buffer. Mix well and use.

   b. If a partial plate is used, prepare enough SA-HRPO Working Dilution as shown below:

No of Tests	**SA Buffer (mL)**	**SA-HRPO (µL)**
24	5.0	5
48	10.0	10
72	15.0	15

   c. Discard unused portion at the end of the day.

6. TMB Diluent. Store at 2-8^{0}C. Note manufacturer's outdate.

   TMB Reagent in dimethyl sulfoxide. Store at 2-8^{0}C. Note manufacturer's outdate. Prepare TMB-substrate Solution as follows:

   a. Within 15 minutes prior to use prepare the TMB-Substrate Solution. For a complete 96 well plate add 21 mL of the TMB Diluent into a clean, disposable plastic container and add 210 µL of TMB Reagent. Mix well and use.

   b. If a partial plate is used, prepare enough TMB-Substrate as follows:

No of Tests	**TMB Diluent (mL)**	**TMB Reagent (µL)**
24	5.0	50
48	10.0	100
72	15.0	150

   c. Discard unused portion at the end of the day.

   Note: TMB-Substrate Solution should appear colorless and, when combined with CSR-1 Solution, should have an absorbance value less than 0.050 at 450 nm 450/570 nm when compared wit a distilled water blank.

8. Lysis Buffer. Store at 2-8^{0}C. Note manufacturer's outdate.

9. Wash Buffer. Store at 2-8^{0}C. Note manufacturer's outdate. Prepare Working Wash Buffer as follows:

   a. To prepare 700 mL of Wash Buffer, dilute 35 mL of 20X Wash Buffer with 665 mL of distilled water.

   b. Discard any unused portion at the end of the day.

10. Coulter Stop Reagent-1 (CSR-1) (4N H_2SO_4). Store at 2-30^{0}C. Note manufacturer's outdate.

B. Reagents included in Coulter ICD-Prep Kit (PN 6604709) include:

1. Glycine Reagent. Store at 2-8^{0}C. Note manufacturer's outdate.

2. Tris Reagent. Store at 2-8^{0}C. Note manufacturer's outdate.

3. Incubation bags.

C. Reagents required but not provided:

1. 5% Hypochlorite solution (household bleach) diluted 1/100 or appropriate disinfectant.

2. Deionized or distilled water.

3. Standards and controls for the assay provided by the Virology Quality Assurance Laboratory (VQA):

   a. VQA SQC (Serum Quality Control). A set of five concentrations. Store at -80^{0}C.

      1) Just prior to set up, thaw 1 vial of each of the 5 concentrations.
      2) Mix well and use.

   b. ICD p24 Positive Control. Once reconstituted, store at 2 -8^{0}C for up to 2 weeks.

      1) Reconstitute the lyophilized ICD positive control with 2.0 mL of dH_2O.
      2) Gently mix by inversion and allow 15 minutes for the reagent to go into solution.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Micropipet(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volume.
Multichannel pipette(s) capable of delivering 10 µL, 20 µL, 50 µL, 200 µL volumes
Disposable pipette tips suitable for the above pipettes
Disposable reagent reservoirs
Uncoated 96 well microtiter plate
Strip holder reaction plate
Incubator without CO_2 capable of maintaining 37^0C +/- 1^0C
Timer capable of measuring times up to 60 minutes
Centrifuge
Graduated cylinders and beakers
Serological pipettes
ELISA microtiter plate washer with waste trap and vacuum source
ELISA microtiter plate reader capable of measuring absorbance at 450 nm with reference at 570 nm

V. PROCEDURE

A. ICD Sample Preparation

1. Bring all reagents and samples to room temperature.

2. Create an ICD EIA template in the virology data-management software (see software manual)

3. Dispense 30 µL of Lysis Buffer to each well that will receive either SQC, V100, ICD p24 Positive Control, or patient specimen as noted on the template. The ICD specimen pretreatment is set up using an uncoated microtiter plate!

4. Dispense 100 µL of SQC, ICD Positive Control or patient specimens as noted on the template. The ICD Positive control will be treated with Glycine Reagent or Neutral Buffer) as described in step 5., resulting in Treated ICD Positive Control, T100, and Untreated ICD Positive Control, U100.

5. Dispense 100 µL of Glycine Reagent to each of the wells except the U100 (ICD Positive control in which you add 100 µL of Neutral Buffer) with a multichannel pipette. Mix the reagents in the wells by filling and dispensing sample and Glycine reagent five times. Avoid foaming by not fully dispensing pipette tip contents when mixing. Change tips before preceding to the next strip.

6. Carefully seal the plate with an adhesive plate cover. Place it in the incubation bag and seal the bag. Incubate the plate at 37^0C for 90 mins.

7. Remove the plate from the incubator and remove the plate cover. Dispense 100 µL of Tris Reagent to each of the wells except the U100 (ICD Positive control in which you again add 100 µL of Neutral Buffer) with a multichannel pipette. Mix the reagents in the wells by filling and dispensing sample and Glycine reagent five times. Avoid foaming by not fully dispensing pipette tip contents when mixing. Change tips before preceding to the next strip. (Note: Samples may now sit for up to one hour before proceeding to the next step, if desired.)

8. Position the required number of HIV-1 p24 Antibody-coated Microtiter Strips in the strip holder reaction plate (8 wells per strip). If fewer than 12 strips are needed, use uncoated strip(s) in the remaining positions when using a 96 well plate washer. Using a multichannel pipette transfer 200 µL of all wells to the corresponding wells of the coated microtiter strip reaction plate.

9. Carefully seal the plate with an adhesive plate cover. Place it in the incubation bag and seal the bag. Incubate the plate at 37^0C overnight (18-24 hrs.).

B. HIV-1 p24 Antigen Assay

1. After overnight incubation, remove the reaction plate from the incubation bag and carefully remove the plate cover and discard.

2. Wash as follows: Aspirate the solution from the wells. Add 300 µL of Wash Buffer Working Dilution to each well. Allow wells to soak for 25-30 seconds. Aspirate the solution from the wells. Wash five (5) more times for a total of 6 washes. After the final wash step, grasp the plate firmly along the edges, invert plate over absorbent paper and tap the plate gently to remove any remaining liquid.

Important: The time between the wash step and the next reagent must be less than five (5) minutes.

3. Add 200 µL of reconstituted Biotin Reagent to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 1 hour $\pm$ 5 minutes.

4. Wash as described above.

5. Add 200 µL of SA-HRPO Working Dilution to all testing wells, except the substrate blank well. Cover the plate using a new adhesive plate cover. Incubate at 37^0C $\pm$ 2 for 30 $\pm$ 2 minutes.

6. Wash as above.

7. Add 200 µL of TMB-Substrate Solution to all wells. Cover the plate using a new adhesive plate over. Incubate at room temperature (15-30^{0}C) for 30 $\pm$ 2 minutes.

8. Add 50 µL of CSR-1 to all wells.

Important: Add CSR-1 to the wells in the same sequence and at the same rate of speed that the TMB-Substrate Solution was added.

9. Read absorbance at 450 nm (reference at 570 nm if dual wavelength is available) within 30 minutes of adding CSR-1 to the wells.

VI. CALCULATIONS

The HIV-1 p24 antigen concentrations may be generated from a virology data-management software program developed for the Division of AIDS (DAIDS) to ensure data integrity of both QA and test specimens. A weighted linear least squares method using the VQA SQC concentrations is used to estimate HIV-1 p24 antigen concentration.

VII. QUALITY CONTROL

The absorbances obtained form the spectrophotometer may be transferred into the virology data-management software program. The software program incorporates two QC check programs, Cum Sum and Levy Jennings. These two programs review the absorbance of the VQA SQC and the Treated and Untreated ICD Positive Control and compare them to established standard deviation ranges. These ranges are determined by the testing laboratory and is reflective of values unique to each laboratory. The software will flag values that fall outside of the laboratory's standard deviation range. The technician must determine the significance of the out of range QC and resolve the situation.

VIII. PROCEDURAL NOTES

The incubation at 37^{0}C during the ICD Sample Preparation step is critical. If the temperature goes above 38^{0}C, coagulation of the samples may occur.

Occasionally, samples will produce a small amount of insoluble material after the overnight incubation. Aspiration of all wells before placing the plate in the washer will eliminate clogging of the plate washer.

If a sample gels completely and the well still contains visible coagulated serum proteins after washing, the results should be considered invalid and the sample retested.

IX. REFERENCES

Coulter HIV-1 p24 Antigen Assay package insert and all references within.

Coulter ICD-Prep Kit package insert and all references within.